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J. Craig Venter™
INSTITUTE

Rapid parallel screening for strain optimization

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Performing Organization:	J. Craig Venter Institute 9704 medical Center Drive Rockville, MD 20850 USA
Principal Investigator:	Chuck Merryman

Abstract

Progress has been made on identifying biosensors that will be used to report on the fermentation yields of industrially relevant biological compounds. Screening of the desired chemicals has been largely completed. Microbes that can utilize these compounds as their sole carbon source have been isolated and archived. Phylotyping of the isolated microbes is nearly finished and genomic DNA has been prepared for sequencing.

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Summary

In this reporting period, we have completed the effort to screen the final 20 compounds from the initial set of chemicals. This set of 20 chemicals is more cumbersome than many of those already processed due to their physical properties (poor water solubility, etc). Most of these compounds were successfully used to isolate microbes that can utilize the chemicals as their sole carbon source.

In total, almost 70 compounds have been paired with microbes as suggested by colony morphology. We have completed clonal purification, generated freezer stocks, phylotyped, and isolated genomic DNA for these. Growth screens for a final batch of 33 chemicals are underway and we expect to have these ready for sequencing in a couple of weeks.

Except for the last batch of chemicals, phylotyping has been completed and we are currently analyzing the data. Genomic DNA from the isolated organisms has been prepared and will be submitted for sequencing on the Illumina HiSeq platform.

Introduction

The overall goal in this contract is to link cell-based production to cell survival and thereby make the engineering of new microbial strains that produce industrially relevant biochemicals routine. Recent synthetic biology techniques can make billions of variant cells. Although, many potentially informative mutants are easily made, product yield can only be determined in a few of these. The majority of industrially relevant biomolecules are not chromophores, naturally discernible, or otherwise easily detected. Nevertheless, genetic circuits are capable of linking chemical production to discernible signals such as growth or color intensity. Such a system would allow numerous mutants and mutant combinations to be examined quickly. Genetic circuits needed to screen mutant populations in parallel rely upon the the availability of an appropriate biosensor that activates a reporter gene in a product dependent fashion. In this project, genes for two-component and one-component signaling systems (that respond to industrially relevant biomolecules) are identified using microbial growth assays, sequencing, and quantitative PCR (qPCR). To demonstrate that such sensors can be used to maximize product yield, one sensing system will be further engineered. We will reformat this sensor so that it drives expression of a reporter such as an antibiotic resistance marker. This sensor/resistance cassette, and a biosynthetic pathway capable of producing the molecule to which the sensor responds, will be placed within a heterologous host that does not have an overlapping pathway. Basal synthesis of

the targeted chemical (by the orthogonal biosynthetic pathway) activates the sensor and increases transcription of the resistance marker (i.e. reporter). In other words, the fermentation product is also the sensor ligand and thus, biosynthesis drives production of the reporter and cell survival. Antibiotic levels in the media will be adjusted so that basal product yield, and hence basal marker activity, is insufficient for survival. Targeted, genome-wide and barcoded alterations to the host genome will then be installed. Variants with better and better chemical production survive by virtue of their ability to withstand increasing antibiotic challenge.

Methods, Assumptions and Procedures

We have continued with the same approach for isolating microbes. Well composted manure samples and pond sediment were diluted 1:5 (w/w) with water and extensively macerated with a spatula. Heavy material was allowed to settle to the bottom of the tube. The aqueous layer was removed and diluted 100-fold with water. Ten microliter aliquots were then distributed in 15 ml culture tubes. Enrichment broths were made using 0.1 percent carbon source in M9 mineral salts media. One-hundred microliters of a 1% solution of the target biochemical (i.e. carbon source) was added to 0.9 ml of M9 salts. A one-percent slurry was used for insoluble solids. Insoluble liquids were used neat and 100 microliters was applied to the mineral salts solution. Tubes containing volatile compounds were sealed with screw-capped lids and opened occasionally. Enrichment cultures were incubated at room temperature and shaken at 200 rpm for 60 hours. Two-microliters of each culture were withdrawn and directly pipetted on the top of a glass bead in each sector of a square culture plate comprised of 48 individual wells. These trays were shaken vigorously to distribute microbes over the media surface. Solid media contained Luria broth, glucose, glycerol, and succinate. As colonies developed they were picked and transferred to a second and third enrichment culture containing the appropriate carbon source and M9 mineral salts. Archived versions were stored at -80C in broth or broth with 15% glycerol. Finally, agar plates using the carbon source and mineral media were also generated, sealed and stored at 4C. Slurry's were formed within the media and insoluble liquids were spread on the surface of plates used for storage.

Phylotyping was performed by amplifying 16S rRNA with appropriate primers, cloning the products and Sanger sequencing. Genomic DNA will be submitted for sequencing in the middle of December.

Results and Discussion

Nearly 70 microbes have been selected based growth properties. Seventy-five chemicals have been used as carbon source. In addition to M9 salts supplemented with a target chemical, each of the isolated microbes can utilize a standard carbon source such as glucose, glycerol, or succinate. This is necessary for evaluating differential expression and the identification of transcription factors that likely bind to an alternative carbon source. A final batch of 33 chemicals is being processed. In the next few weeks we expect to have about 96 microbe-chemical pairs. Regardless of the exact count, we will restrict ourselves to a maximum of 96 sequenced organisms as that is convenient in our sequencing pipeline.

Conclusions

The results continue to indicate that any chemical made by one organism is likely to be used as food by another. Microbes typically utilize the most efficient carbon source available (glucose often being the preferred substrate). More exotic carbon sources are generally subject to catabolite repression and systems for their utilization are activated after preferred carbon sources are exhausted. Besides catabolite repression, sensors are often employed so that a microbe can activate the appropriate degradation pathway for a non-preferred carbon source. Nevertheless, how often sensor systems are used cannot currently be estimated because negative results rarely appear in the literature. We have nearly finished generating an extensive data set that will allow us to provide such an estimate. The next step is to use low cost next-generation

sequencing and functional genomics to unequivocally identify sensors and how often they are employed for growth on non-standard carbon sources. This will set the stage for downstream work on the overproduction of such molecules.

Planned Activities for the Next Reporting Period

During the next reporting period we will select our final set of microbes and sequence them. We will begin the bioinformatic analysis of the sequenced genomes should sequencing be completed early.

Program Financial Status

	Planned Expend	Actual Expend (Cumulative to Date)	% Budget Completion	At Completion	Latest Revised Estimate	Remarks
Task 1	$59,251	$59,251	100.0%	$59251	$59,251	Completed
Task 2	$183,957	$42,057	22.9%	N/A	$183,957	In progress
Task 3	$124,706	$0.00	0.0%	N/A	$124,706	N/A
Cumulative	$367,914	$101,308	27.54%	N/A	$367,914	N/A

There is no management reserve or unallocated resources.

Based on the currently authorized work:

- Is current funding sufficient for the current fiscal year? Yes
- What is the next fiscal year funding requirement at current anticipated levels? The budgeted amount for Year 2 of the project is $396,905.25.
- Have you included in the report narrative any explanation of the above data and are they cross-referenced? Not applicable; current funding is sufficient for the current fiscal year.